Remarks

Applicants' response is timely filed on October 4, 2001 with a 1-month extension of time to the Office Action mailed June 5, 2001.

In addition, two typographical errors have been attended to in the specification. No substantive change has been made by amendment, and no new matter has been added to the application.

Response to the rejection under 35 USC §112, second paragraph.

The Examiner has rejected claims1-24 under 35 USC §112, second paragraph Applicants appreciate the comments made by the Examiner regarding 35 USC §112, second paragraph, in light of which Applicants have cancelled claims 9 and 11-12, amended claims 1-8, 10, 13-18 and 20, and added five new claims (claims 21-25). Moreover, the elements of the now cancelled claims 11 and 12 have been added to claim 3, thus the dependencies on claim 3 are now correct. Support for the covalent crosslinking of the copolymers in the polymersomes may be found at least in section D of the Detailed Description, beginning on page 30. In light of the amendments, the Examiner's objection to claim 11 is now moot.

Claims 14 and 16 have been amended to refer to a Markush group of "materials," rather than "compositions," although Applicants' original reference was intended to mean a "composition of matter" as opposed to a qualitative chemical state. The term "materials" was selected rather than the term "compound" suggested by the Examiner because it was consistent with the terms used in claim 15. Thus, the claims encompass a polymersome encapsulating "at least one material selected from the group consisting of drug, therapeutic compound, dye, indicator, waste product, heavy metal, biocide, nutrient, sugar, vitamin, mineral, protein or protein fragment, salt, electrolyte, gene or gene fragment, product of genetic engineering, steroid, adjuvant, biosealant, gas, ferrofluid, and liquid crystal."

The Examiner has asked Applicants to define and distinguish the following terms used in claims 14 and 16 regarding the encapsulated materials: "drug" as distinguished from a "therapeutic compound" and a "steroid;" "nutrient" as distinguished from a "sugar;" "salt" distinguished from "electrolyte." Also the Examiner has asked that the terms "biosealant" and "ferrofluid" be defined as they are used in the present invention.

Many of the materials that can be encapsulated in the polymersomes of the present invention are identified and listed in the specification in Section C, beginning at page 29 and continuing on page 30. The terms questioned by the Examiner are not expressly defined within the application since they are terms commonly understood by one of ordinary skill in the art in the field of the invention, and thus need not be expressly defined to permit such an individual to practice the invention. The terms have the following meanings (Grant & Hackh's Chemical Dictionary 5th Edition, 1987).

Drug means "a medicinal substance ... classified according to composition or constituents, structure or physical features, effect and use, origin and source."

Therapeutic compound is essentially the same as a *therapeutic agent* which means "a remedy or substance used to alleviate disease, pain or injury."

Steroid is the generic name for a family of lipid compounds comprising the sterols, bile acids, cardiac glycosides, saponins, and sex hormones."

Nutrient means " a food for a body organism or cell."

Sugar means "a sweet carbohydrate."

Salts mean "substances produced from the reaction between acids and bases; a compound of a metal (positive) and a non-metal (negative) radical."

Electrolyte means "a substance that dissolves into 2 or more ions, to some extent, in water."

Biosealant is derived from the term *seal* meaning "material placed around joints to prevent the passage of liquids or gases," in this case, the material is a biological material.

Ferrofluid is derived from the term *ferro-* meaning "prefix indicating metallic iron," in this case a fluid containing metallic iron.

Thus, Applicants have used a number of interrelated terms to define the encapsulated materials to avoid indefiniteness that could result if Applicants tried to claim all materials or tried to claim such broad categories of materials that the practitioner could not accurately determine the subject matter of Applicants' invention. The terms "drug" and "therapeutic compound" are related but distinguishable. A drug can include prophylactic materials and other materials that may not necessarily be considered therapeutic. Certain therapeutic compound, such as naturally occurring herbs or enzymes are not necessarily considered "drugs" by the

traditional medical community, yet they are considered to be "therapeutic" by Chinese herbalists and other alternative medical providers. Consequently, the terms overlap, but are also distinct.

Similarly, as defined, "steroids" encompass only one subset of materials, specifically a family of lipid compounds. However, although the term overlaps with "drugs," not all steroids are necessarily drugs, and although the term overlaps with "therapeutic compounds," not all steroids are therapeutic because some are prophylactic or in some circumstances even harmful. Consequently, the terms overlap, but are also distinct

The terms "nutrient" and "sugar" are also related but distinguishable. There is no question that a sugar can be considered a food, but is also is referred to as a simple chemical carbohydrate formulation, which may be neither edible or considered to have food value. In fact, when consumed by diabetic patients, sugar is not only not an acceptable food, it is actually harmful to such an individual. Therefore, both terms are appropriately listed in the Markush group because, although overlapping in certain situations, the terms are also distinct.

A "salt" may be an "electrolyte" in certain aqueous situations, but salts are necessarily the product of an acids/base reaction or of positive/negative radicals. Similarly, a salt can in some circumstances be considered a nutrient, although not a food. Yet, in those circumstances, it may not act as an electrolyte. Therefore, both terms are appropriately listed in the Markush group because, although overlapping in certain situations, the terms are also distinct.

The term "biosealant" is well recognized in the are of biological tissue adhesives and fibrin-based sealants to control hemostasis at a wound or site of injury. Such materials often contain other compounds or compositions, such as growth hormones to enhance cell delivery to speed healing, analgesics or anesthetics to control pain at the site, or antibiotics to control infection. Thus, such biosealants could be significantly improved by the addition of Applicants' polymersomes to provide the controlled release of any additive that may be contained in therein (see specification at, *e.g.*, page 36, lines 10-21). Similarly, one of ordinary skill in the art could also deliver an iron-based material, such as hemoglobin, *i.e.*, a "ferrofluid" from the polymersomes or to remove such materials from an environment.

In light of the foregoing definitions and descriptions, the Examiner's rejection is moot.

The Examiner further asks about the electroforming step as recited in claim 17. "Electroformation" is a term well known in the art to which Applicants' invention applies, and thus, it requires no express definition. However, Applicants respectfully direct the Examiner to

the specification at Section A (Preparation of Polymersomes) beginning at page 24, wherein Applicants provide a detailed description of Film Rehydration, beginning at page 25, Bulk Rehydration, beginning at page 26, line 1, and Electroformation, beginning at page 26, line 9. In light of Applicants' description and exemplification of "electroformation" the practitioner reading the specification would clearly understand the term and what is meant by the preparation process. Consequently, the Examiner's rejection is moot.

Regarding the Examiner's question regarding "modulating the composition of the membrane" in claim 18, Applicants are unclear why the Examiner raises the issue. The method clearly sets forth an active step – modulating – in the proper form. The term is well understood to mean altering or changing, *e.g.* increasing or decreasing a component or characteristic of the membrane (see, *e.g.*, specification at page 8, beginning at line 22, wherein methods for controlling release of an encapsulated material into or out of a polymersome is discussed). Further, Applicants provide methods for determining the changes in various moduli of deformation and strength, including thickness, that would affect transport of an encapsulated material, *e.g.* at page 22-23, 32 and 38-41. The effects of modulating cross-linking and permeability are discussed, *e.g.*, at page 31-32. The effects of modulating temperature stability are discussed, *e.g.*, at page 33-34. Moreover, Applicants have provided detailed Examples and calculations to clearly teach the practitioner how to modulate or alter the composition of the membrane to achieve various characteristics which affect the transfer of materials into and out of the polymersomes, depending on size and composition.

Applicants' are perplexed as to what more the Examiner could be seeking in the way of clarification of the variations available to Applicants based upon their description of the invention. However, if the Examiner will offer some suggestion of the language or limitations he believes are necessary, Applicants will reevaluate claim 18 in terms of such proposed language.

Finally, regarding the Examiner's query as to whether claim 20 refers to a product or a process claim, Applicants claim a product, which is obtained by a particular process in which the membrane is formed around an oil micro-droplet in water. The claims has been amended accordingly, and the objection is now moot.

Accordingly, all of the objections and rejections under 35 USC §112, second paragraph has been rendered moot, and Applicants respectfully request that the entire rejection be withdrawn.

Response to the rejections under 35 USC §102.

The Examiner has rejected claims 1-4 and 9-15 under 35 USC § 102(b) as anticipated, over Henzelwood (*Macromolecules,* 1998) or Hajduk (*J. Phys. Chem.*, 1998) or Ding (*J. Phys. Chem.*, 1998) or Cornelissen (*Science*, 1998) or Fendler (*Science*, 1998). In making this rejection, the Examiner states that each of the cited references teaches polymeric vesicles having a membrane, wherein according to the abstracts of each, the polymers are diblock polymers. In view of this work, the Examiner has rejected Applicants' invention.

However, contrary to the Examiner's interpretation of the prior art, none of the cited references teach polymeric vesicles. Consequently, none anticipate Applicants' invention.

"Vesicles," as the term is used in the present invention are defined at page 13, beginning at line 25. They are "essentially semi-permeable bags of aqueous solution as surrounded (without edges) by a self-assembled, stable membrane composed predominantly, by mass, of either amphiphiles or super-amphiphiles which self-assemble in water or aqueous solution." The membrane of a vesicle functions as a barrier to maintain a difference in composition and an osmotic balance between the interior of the vesicle and the exterior. At page 14, lines 19-21, an "encapsulating membrane" is defined as functioning to compartmentalize "by being semi- or selectively permeable to solutes, either contained inside or maintained outside of the spatial volume delimited by the membrane." Thus, a vesicle is a capsule in aqueous solution, which also contains aqueous solution. However, the interior or exterior of the capsule could also be another fluid, such as an oil or a gas. A "capsule," as the term is used in the present invention, is defined at page 14, lines 23-25, "the encapsulating membrane plus the space enclosed within the membrane."

In a detailed review of the cited Hajduk, 1998 reference, the behavior taught is the self-assembly of block copolymers – but Applicants can find no description or mention of "vesicles" in the title, abstract, or results; nor is there any other discussion or demonstration of such. The authors simply describe the formation of bulk lamellar phases and other non-vesicular phases in aqueous solutions that can be regulated by both synthetic tuning of polymer chemistry and

physical variables, such as concentration and temperature (see page 5, lines 13-17). Contrary to the description provided in the specification at page 14, that a vesicular structure must be comprised of a membrane, which separates an internal solution from an external solution, Hajduk provides no indication of any diblock formation into a vesicle of any type. As a result, Hajduk cannot anticipate the vesicles of the present invention, nor can it anticipate the invention as a whole.

Similarly, Henselwood, 1998 and Ding, 1998 fail to mention or describe "vesicles" in the title, abstract, or results; nor is there any other discussion or demonstration of such. In each case, the authors simply describe the formation of spherical micelles of diblock copolymers, wherein the micelle has a diameter comparable to amphiphile dimensions (see, present application at page 5, lines 1-5). However, the disclosed structures are altogether lacking in any sort of membrane that separates an internal solution from an external solution as is defines a vesicle. Hence, neither Henzelwood, nor Ding, anticipate the vesicles of the present invention. Thus, neither can anticipate the invention as a whole

Fendler, 1984, teaches only small amphiphiles (lipid-like in molecular weight) with no more than four covalently crosslinkable bonds. However, neither Fendler, nor any other reference on such amphiphiles, describes vesicles of the type disclosed in the present invention, which remain intact following either a cycle of dehydration and rehydration, or exposure to organic solvents (see, present application at page 3, lines 1-9). By comparison, polymersome membranes made from super-amphiphilic copolymers that incorporate an effective number of crosslinkable groups per copolymer are crosslinked into a contiguous, semi-permeable membrane.

As proof of thorough crosslinking in the vesicles of the present invention, polymersomes with crosslinked membranes remain as intact vesicles, maintaining their encapsulated contents, after at least one cycle of dehydration and rehydration or exposure to organic solvents, such as chloroform (see, *e.g.*, FIG. 10, FIG. 11 or the specification at page 33, lines 10-14). Consequently, the amphiphiles taught by Fendler do not anticipate the crosslinked membrane of the polymersome vesicles of the present invention, nor can it anticipate the invention as a whole

In support of the crosslinking of the polymersome membranes of the present invention, Applicants attach hereto a recently submitted manuscript by the inventors. This manuscript,

prepared after the filing date of the invention, is not prior art; rather it is submitted to describe the present understanding of the art in light of the present invention.

Finally, Cornelissen *et al.*, 1998, did not use strictly synthetic super-amphiphiles. The starting material in the polymer synthesis by Cornelissen *et al.* included di-alanine polymer, a naturally derivable dipeptide. Cornelissen *et al.* only compared their compounds to helix-forming proteins. They make no comparison to lipid or other vesicle-forming amphiphiles.

Importantly, Cornelissen *et al.* show only vacuum-dried Transmission Electron Microscope (TEM) images of what are seen to be rods, helices, and loops – the images are not of vesicles or vesicular systems. The authors failed to teach, suggest, or in any way demonstrate that their polymeric synthesis produced intact synthetic super-amphiphilic vesicles in aqueous or any other solution, or that their product could remain intact under dehydration/rehydration conditions or in the presence of an organic solvent. Thus, the cited reference fails to anticipate the invention.

Consequently, it is clear that each of the cited references fails to anticipate Applicants' invention under 35 USC § 102(b) since none teach the polymeric vesicles of the present invention. Accordingly, Applicants respectfully request that the rejection be withdrawn.

The Examiner has also rejected claims 1-4, 6, and 9-18 under 35 USC § 102(a) as being anticipated by Hentze (*Macromolecules,* 1999). As above, the Examiner reads Hentze as teaching polymeric vesicles having a membrane, wherein the polymers are described in the abstract as diblock polymers. However, as explained above with regard to the cited Hajduk, 1998 reference, Applicants can find no description or mention of "vesicles" in the title, abstract, or results; nor is there any other discussion or demonstration of such. The authors simply describe the formation of bulk lamellar phases and other non-vesicular phases in aqueous solutions that can be regulated by both synthetic tuning of polymer chemistry and physical variables, such as concentration and temperature (see page 5, lines 13-17).

Contrary to the description provided in the specification at page 14, that a vesicular structure must be comprised of a membrane, which separates an internal solution from an external solution, Hentze provides no indication of such. As a result, Hentze cannot anticipate the vesicles of the present invention. In a detailed review of the cited reference, Applicants can find no description or mention of "vesicles" in the title, abstract, or results; nor is there any other discussion or demonstration of such. The authors simply describe the formation of bulk lamellar

phases and other non-vesicular phases in aqueous solutions that can be regulated by both synthetic tuning of polymer chemistry and physical variables, such as concentration and temperature (see page 5, lines 13-17.)

Contrary to the description provided in the specification at page 14, that a vesicular structure must be comprised of a membrane, which separates an internal solution from an external solution, Hentze provides no indication of such. As a result, Hentze cannot anticipate the vesicles of the present invention, or the invention as a whole. Accordingly, Applicants respectfully request that the rejections be withdrawn.

The Examiner has also rejected claims 1-2, 7-9, and 14-18 under 35 USC § 102(a) as being anticipated by Liu (*Macromolecules,* 1999). As above, the Examiner reads Liu as teaching polymeric vesicles having a membrane, and acryloylphospholipids which are cross-linked (see abstract). However, as explained above with regard to the cited Henzelwood, 1998 reference, although Liu teaches cross-linking polymerization of hydrated amphiphiles in monolayers, bilayers and nonlamellar phases, Liu fails to mention or describe the formation of "vesicles" in the manuscript. Instead, the authors simply describe spherical micelles having a diameter comparable to amphiphile dimensions (see, present application at page 3, lines 3-9). However, the disclosed structures are altogether lacking in any sort of membrane that separates an internal solution from an external solution as is defines a vesicle. Hence, Liu fails to anticipate the vesicles of the present invention, or the invention as a whole. Accordingly, Applicants respectfully request that the rejections be withdrawn.

When examined, each of the cited prior art references quite simply operates in a completely different manner from the present invention. Thus, the prior art fails to define every element of Applicants' invention, meaning that the cited references fail to anticipate the invention. Consequently, Applicants respectfully request that in light of the foregoing, the rejections under 35 USC § 102 (a) and (b) be reconsidered and withdrawn.

Response to the rejection under 35 USC §103.

The Examiner has rejected claims 5 and 14-20 under 35 USC § 103 as obvious, over Henzelwood (*Macromolecules,* 1998), Ding (*J. Phys. Chem.*, 1998), Fendler (*Science*, 1998) or Hentze (*Macromolecules,* 1999) for the above-stated reasons. In making this rejection, the Examiner states that each of the cited references suggests potential applications of polymeric

vesicles for drug delivery. In addition, the Examiner states that the criticality of the triblock polymer is not apparent from Applicants' invention since it appears that the amphiphilic nature of the polymer is the determining factor. Hence, the Examiner has rejected Applicants' invention.

However, contrary to the Examiner's comments, and as described in detail above, none of the cited references discloses or even suggests the formation of a polymeric vesicle, let alone links how such non-disclosed vesicles could be used for drug delivery. As a result, for the above identified reasons that the cited references were alone unable to anticipate the present invention, they fail to render Applicants' invention obvious. Even if combined, and further combined with the knowledge of the art at the time of the invention, gaps remain unfilled that could make Applicants' invention obvious to one of ordinary skill in the art with any expectation of success without undue experimentation. These deficiencies cannot been met by the combining of the references that each failed to stand alone to teach any art of Applicants' invention. Each cited reference fails to teach or suggest polymeric vesicles. Thus, even when combined, they cannot teach the formation of a polymeric vesicle, or Applicants' use thereof; and they cannot render Applicants' invention obvious.

As far as the Examiner's inquiry regarding the triblock versus diblock copolymers, what is required for the definition of polymersome in the present invention, is a super-amphiphilic block copolymer containing either two or three blocks.

Accordingly Applicants again point to the overwhelming differences between the prior art configurations and that of the present invention, none of which are met by the references relied upon by the Examiner. The present invention quite simply operates in a completely different manner from the prior art, and produces vesicles that have a multitude of uses, none of which have been previously possible. Thus, the prior art fails to render Applicants' invention obvious, and Applicants respectfully request that in light of the foregoing, the rejection under 35 USC § 103 be reconsidered and withdrawn.

In sum, Applicants assert that all pending claims are in condition for allowance, and respectfully request that allowance be granted at the earliest date possible. Should the Examiner have any questions or comments regarding Applicants' amendments or response, she is asked to contact Applicants' undersigned representative at (215) 575-7034.

Respectfully submitted

Date: October 4, 2001

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Version with markings to show changes made

In the specification:

Paragraph at page 4, lines 17-29 has been amended as follows:

Many wholly or partially synthetic, amphiphilic molecules are also significantly larger (in molecular weight, volume, and linear dimension) than phospholipid amphiphiles, and have therefore been called "super-amphiphiles" (Cornelissen *et al., Science* 280:1427 (1998)). Cornelissen *et al.* used polystyrene (PS) as a hydrophobic fraction in their series of synthetic block copolymers designated PS40-b-(isocyano-L-alanine-L-alanine)y. For y = 10, but not y = 20 or 30, loop structures, referred to as small collapsed vesicles, having ~~with~~ diameters ranging from tens of nanometers to several hundred, and a bilayer thickness of 16 nanometer were mentioned as existing under a single acidic buffer condition (0.2 mM Na-acetate buffer, pH 5.6). However, bilayer filaments and superhelical rods existed, without explanation, under the same solution conditions, thus making the stability of the collapsed vesicles, relative to the other microstructures, highly uncertain for the studied dipeptide-based copolymer ~~polymer~~. Furthermore, no demonstration of semi-permeability was reported, and reasons for apparent vesicle collapse were not given, further raising questions of vesicle stability.

Paragraph at page 5, lines 10-20 has been amended as follows:

Both ~~amphiles~~ amphiphiles and super-amphiphiles can exist in a broad variety of microphases and bulk phases that include not only lamellar, but also hexagonal, cubic, and more exotic phases (see review by Lipowsky and Sackmann, in Handbook of Biological Physics, 1995; Bates, *Science* 251:898 (1991). Based on the work of Hajduk *et al.* (see, *J. Phys. Chem. B* 102:4269 (1998)), the ability of super-amphiphilic block copolymers to form lamellar phases in aqueous solutions can be regulated by both synthetic tuning of polymer chemistry and physical variables ~~like~~, such as concentration and temperature. Evidence has now accumulated that in dilute solutions certain diblock copolymers, such as polyethyleneoxide-polyethylethylene (PEO-PEE, wherein PEO is structural equivalent to PEG), can form not only worm-like micelles (Won *et al., Science* 283:960-3 (1999)), but also unilamellar vesicles (Discher *et al., Science* 284:1143 (1999)).

Paragraph at page 40, lines 13-21 has been amended as follows:

Related to the length scales above, the root ratio of moduli, $(K_b/K_a)^{1/2}$, is generally recognized as providing a proportionate measure of membrane thickness (see, *e.g.*, Handbook of Biological Physics, *supra*; Bloom *et al.,* 1991; Needham *et al.*, 1996, chap. 9; and Petrov *et al., Prog. Surf. Sci.* 18:359 (1984)). For the presently described polymersome membranes, $(K_b/K_a)^{1/2}$ = 1.1 nm on average. By comparison, fluid bilayer vesicles of phospholipids or phospholipids plus cholesterol, have reported a ratio of $(K_b/K_a)^2$ = 0.53 to 0.69 nm (Evans *et al.*, 1990; Helfrich *et al.*, 1984). Typically, the fluid bilayer vesicles of ~~phospholipids~~ phospholipids plus cholesterol have a higher K_a than those of phospholipid alone.

Section heading at page 45, line 19, has been amended as follows:

Example 3: Polymersomes from Amphiphilic Triblock and ~~Multi-BlockCopolymers~~ Multi-Block Copolymers

In the claims:

Claims 9 and 11-12 have been cancelled.

Claims 1-8, 10, 13-18 and 20 have been amended as follows:

1. (Amended) A polymersome vesicle comprising a semi-permeable, thin-walled encapsulating membrane, wherein the membrane is formed in an aqueous solution, and wherein the membrane comprises one or more synthetic super-amphiphilic molecules.

2. (Amended) ~~A~~ The polymersome vesicle of claim 1, wherein at least one super-amphiphile molecule is a block copolymer.

3. (Amended) The polymersome vesicle of claim ~~1~~ 2, wherein ~~at least one super-amphiphile molecule is a block copolymer and wherein the resulting vesicle is termed a polymersome~~ super-amphiphilic molecules are covalently cross-linked after self assembling into vesicles, and the vesicles remain intact upon exposure to (i) organic solvent, (ii) boiling water, or (iii) dehydration in air or rehydration in aqueous solution.

4. (Amended) The polymersome vesicle of claim 3, comprising a diblock copolymer.

5. (Amended) The polymersome vesicle of claim 3, comprising a triblock copolymer.

6. (Amended) The polymersome vesicle of claim 3, wherein all of the super-amphiphile molecules are block copolymers.

7. (Amended) The polymersome vesicle of claim 3, wherein the vesicle is prepared together with one or more small amphiphiles.

8. (Amended) The polymersome vesicle of claim 7, wherein at least one small amphiphile is a phospholipid.

10. (Amended) The polymersome vesicle of claim 3, wherein at least one block copolymer is selected from the group consisting of polyethylene oxide (PEO), poly(ethylethylene) (PEE), poly(butadiene) (PB), poly(styrene) (PS) and poly(isoprene) (PI).

13. (Amended) The polymersome vesicle of claim 3, wherein the vesicle is biocompatible.

14. (Amended) The polymersome vesicle of claim 3, wherein the polymersome encapsulates at least one ~~composition~~ material selected from the group consisting of ~~a~~ drug, therapeutic compound, dye, indicator, waste product, heavy metal, biocide, nutrient, sugar,

vitamin, mineral, protein or protein fragment, salt, electrolyte, gene or gene fragment, product of genetic engineering, steroid, adjuvant, biosealant, gas, ferrofluid, and liquid crystal.

15. (Amended) ~~The~~ A method of using the polymersome vesicle of claim 3, wherein the method comprises transporting ~~to transport an~~ at least one encapsulatable material to or from the environment immediately surrounding the polymersome.

16. (Amended) The method ~~of using the polymersome~~ of claim ~~13~~ 15, wherein the environment is in a patient, wherein the method further comprises transporting the encapsulatable material ~~to transport~~ to or from ~~a~~ the patient ~~a composition consisting of a drug, therapeutic composition, dye, nutrient, sugar, vitamin, protein or protein fragment, salt, electrolyte, gene or gene fragment, product of genetic engineering, steroid, adjuvant, biosealant and gas to a patient in need of such composition.~~

17. (Amended) The method of preparing the polymersome of claim 3, comprising at least one step consisting of a film rehydrating step, a bulk rehydrating step, or an electroforming step, or any combination thereof.

18. (Amended) A method of controlling the release of an encapsulated material from a polymersome of claim 3, comprising ~~by~~ modulating the composition of the membrane, thereby altering the nature of the enccapsulatable material that may be transported to or from the bulk surrounding the polymersome.

20. (Amended) An encapsulating membrane comprising a semi-permeable, thin-walled encapsulating, amphiphilic membrane~~, wherein~~ prepared by forming the membrane ~~is formed~~ around a droplet of oil in a microemulsion of oil dispersed in an aqueous solution, wherein the membrane comprises one or more synthetic super-amphiphilic molecules.

The following new claims have been added:

-- 21. A method of using the polymersome vesicle of claim 15, wherein the method comprises delivering at least one material encapsulated by the polymersome from the polymersome to the environment immediately surrounding the polymersome.

22. A method of using the polymersome vesicle of claim 15, wherein the method comprises encapsulating at least one material, from the environment immediately surrounding the polymersome, into the polymersome, thereby removing the at least one material from the environment by its encapsulation in the polymersome.

23. The method of claim 16, wherein the method further comprises delivering at least one material encapsulated by the polymersome to the patient, and wherein the encapsulated material is selected from the group consisting of a drug, therapeutic composition, medicament, dye, indicator, nutrient, sugar, vitamin, mineral protein or protein fragment, salt, electrolyte, gene or gene fragment, product of genetic engineering, steroid, adjuvant, biosealant and gas.

24. The method of claim 16, wherein the method further comprises encapsulating at least one material from the patient into the polymersome, thereby removing the material from the patient by its encapsulation in the polymersome, followed by removing the polymersome and the material encapsulated therein from the patient, wherein the encapsulated material is selected from the group consisting of a drug, therapeutic composition, medicament, dye, indicator, nutrient, sugar, vitamin, mineral, protein or protein fragment, salt, electrolyte, gene or gene fragment, product of genetic engineering, steroid, adjuvant, biosealant and gas.

25. The polymersome vesicle of claim 3, comprising a multi-block copolymer. --

Crosslinkable Polymersomes: Membranes with Broadly Adjustable Properties

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Abstract

Massively crosslinked and property–tunable membranes have been fabricated by free radical polymerization of self-assembled, block copolymer vesicles – polymersomes. Similar efforts with crosslinkable lipids would appear frustrated in the past due to at least two factors: limited reactivity and membrane fragility under local stresses of nano-confined crosslinking. We describe here a diblock copolymer of polyethyleneoxide-polybutadiene that has a hydrophilic weight fraction like that of lipids and forms robust fluid phase membranes in water. The polymersomes sustain free radical polymerization of the hydrophobic butadiene, thereby generating a semi-permeable nano-shell. Crosslinked giant vesicles prove stable in chloroform and can also be dehydrated and re-hydrated without rendering the ~8 nm thick membrane core; the results imply defect-free membranes many microns–squared in area. Surface elastic moduli as well as sustainable wall stresses up to 10^3 Atm – orders of magnitude greater than any natural lipid membrane – appear consistent with strong tethering between close-packed neighbors. The enormous stability of the giant vesicles can be tuned down for application: blending in the hydrogenated analog polyethyleneoxide-polyethylethylene modulates the effective elastic constants as well as the rupture strength by orders of magnitude. Results appear consistent with rigidity percolation through a finite-layer stack of two-dimensional lattices. Moreover, below the percolation limit, a regime of hyper-*instability* emerges, reflecting perhaps nanoscale demixing and suggestive of the limitations encountered with low reactivity lipids. The results provide general insights into covalent crosslinking within self-assembled nanostructures.

Introduction

Covalent crosslinking of polymeric assemblies is a long-accepted means of bulk stabilization but is also rapidly emerging as an important approach to bridging the nano-scale world of labile self-assemblies with the meso-scale. As prime examples of massively crosslinked structures, giant nano-rods many microns in length have been reported for at least two block copolymer systems[1,2]. Free-radical crosslinking of EO_m-BD_n (m = 55, n = 45) has been shown, in aqueous dispersions, to give a considerably more elastic material than rods of the non-crosslinked precursor. Other successful examples of self-assemblies that have been driven by polymeric surfactancy and stabilized by cross-linking reaction include aligned nano-wire arrays[3] and optically-patterned thin film mesophases[4]. However, assessing the material nature of inner polymerized nano-structures – with elastic and stability properties no longer dominated by fluid interfaces – can prove a challenge. Simple observations of morphology or else tests of solubilization and heat stability generate initial benchmarks but generally provide little to no insight into critical issues for nano-technology including polymerization-induced strains[2] and defects that limit stability.

Membranes, particularly those that define and delimit vesicles, can serve as sensitive and accessible model systems for understanding many such nano-structure / response relationships[5]. The stability enhancement of self-assembled lipid membranes by polymerization has been investigated for many years [6,7]. Stabilization has to some extent been proved with a range of sub-micron vesicle systems. For instance, O'Brien's group has recently fabricated small vesicles with high degree of polymerization which strongly inhibited detergent induced leakage of entrapped solutes[8]. Furthermore, some pure and mixed component systems are beginning to find use in applications such as oral drug delivery[9]. However, many years of effort suggest that the requisite 2-*D*–reaction is difficult to propagate without the induction of membrane curvature, defects, and rendering over supramolecular length scales[10,11].

An increasing realization of the need for enhanced reactivity[12,13] underscores the limits imposed by 'small', lipid-size membrane constituents. More massive block copolymers considerably broaden the general synthetic approach. Several di- and tri-blocks are now well known to self assemble in purely aqueous solutions into "polymersomes" that range from nano-scale[14,15] to cell-sized giant vesicles with thick as well as extraordinarily tough membranes[16,17]. As a clear consequence of added membrane thickness, copolymers that are now also made to be crosslinkable such as the polyethyleneoxide-polybutadiene (PEO-PBD) diblock elaborated here, necessarily contain many more reactive groups than can be achieved with lipids. Crosslinkable block copolymers, with their intrinsic toughness and high reactivity, are thus predisposed to the generation of highly stable, supramolecular surfaces.

The nature of the chemical crosslinking and the new physical properties that arise at the nano-scale are of general relevance. Giant vesicles reported here offer an alternative and more direct route to understanding meso-material relations. Nano-scale relations between crosslink arrangement and micellar properties seem difficult to elucidate except through a heavy reliance on theoretical models of *bulk* response such as those developed for filamentous actin[18]. With the newly developed vesicular, quasi-two-dimensional systems, various tests of membrane permeation provide molecular-scale insights into defect density while morphological clues that span many length scales generate insights into crosslinking efficiency. Since the micron-size surfaces can be directly characterized by methods of controlled manipulation[e.g.16], crosslink-dependent measures of mechanical properties can be obtained. Polymerization phenomena ranging from 2-*D* rubber elasticity to bond percolation through a non-crosslinkable matrix are thus made accessible, with broad and general implications to mesophase stabilization. Moreover, specific property comparisons are fruitfully made with naturally occurring laterally crosslinked structures, notably the spectrin skeleton of the red cell plasma-lemma. This cell biological structure has clearly been shown critical to biomembrane stability[19] and has also long motivated synthetic efforts

at mimicry[20]. Put all together, what emerges is a coherent picture of nano-structure in relation to collective meso-material properties.

Experimental methods

Block copolymers were synthesized by anionic polymerization as described earlier[2,17]. Vesicles were formed per Fig. 1, and the polybutadiene cores were cross-linked by free radical polymerization in solution. Radicals were generated with the initiator $K_2S_2O_8$ and a redox couple, $Na_2S_2O_5/FeSO_4 \cdot 7H_2O$, largely following Won[2]. As described in Fig.4, the extent of reaction was established by measuring changes in membrane rupture tension. The mechanical properties of both polymersome membranes, the crosslinked and the uncrosslinked, were obtain (at ~23°C) using micropipette aspiration[e.g. 16].

Results

Giant unilamellar vesicles form spontaneously when slivers of bulk EO_{26}-BD_{46} (designated **OB2**) are added to aqueous solutions (Fig. 1A). Such vesicles exhibit several characteristics indicative of a fluid-phase membrane. First, when sheared from their substrate and isolated, vesicles transform morphologically with smooth contours (Fig. 1B). Second, like any lipid bilayer, these uncrosslinked OB2 vesicles dissolve readily when a good solvent such as chloroform is added at only a fraction of a percent. Each molecule in the self-assembled vesicle contains hydrophobic block with forty six double bonds readily available for cross-linking by common means of polymerization. When these giant vesicles are exposed to free radicals in an osmotically-balanced medium, massive cross-linking is achieved. The membrane transformation from liquid to solid state is directly observable upon osmotic deflation of the originally spherical vesicle as creases, folds, wrinkles, and dents proliferate (Fig. 1C). Like deflation of a micron-size rubber ball, these features reflect the fact that molecules cannot significantly rearrange within the surface to relax accumulated strain[21]. At the same time, phase contrast images clearly demonstrate membrane integrity

through sustained retention of small molecule encapsulants such as sucrose (Fig. 1C). Vesicle shape as well as membrane thickness are minimally affected by the crosslinking reagents or the reaction process itself as long as solution osmolarity is held constant.

Cross-linked polymersomes were first tested for stability in chloroform ($CHCl_3$), an excellent solvent for **OB2**. Transfer of a single vesicle into $CHCl_3$ from phosphate-buffered saline (PBS) shows no alteration of vesicle size, shape, volume, or area for as long as the vesicle was held in solvent (Fig. 2A). If chloroform were to significantly swell the hydrophobic core without being strongly opposed by intramembrane crosslinking, the aspirated projection of the vesicle would have lengthened with the increase in membrane area. This is not observed. Moreover, upon transferring the vesicle back to aqueous solution, the vesicle's geometry is unaffected, indicative of full retention of the encapsulated solute, sucrose. Loss of even a few weight percent sucrose from the vesicle into the chloroform would again have lengthened the aspirated projection due to a decrease in vesicle volume during osmotic equilibration[16]. This principle, along with a finite permeability of the crosslinked membrane to water, is illustrated in the bottom panel of Fig. 2A through a final increase in external solute concentration. The encapsulated sucrose is still retained inside the vesicle as again directly imaged by phase contrast microscopy using the refractive index difference between the interior sucrose and the exterior PBS. Stability and integrity of the crosslinked membrane down to the molecular scale are thus confirmed.

Cross-linked **OB2** membranes were next tested for stability through a simple cycle of dehydration–rehydration. A vesicle aspirated into a micropipette is pulled out of aqueous solution (Fig. 2B), across the water-air interface. As the picoliters of encapsulated water evaporate through the increasingly wrinkled membrane, the vesicle collapses. Subsequent return of the semi-dehydrated vesicle to aqueous solution leads, however, to rapid rehydration and vesicle swelling as illustrated in the bottom panel of Fig. 2B. The phase contrast again indicates retention of encapsulated solutes. Similar experiments in bulk show that cross-linked vesicles can be dried, stored at room temperature for days and then

rehydrated to their original average diameter and volume. Vacuum drying allows direct imaging by scanning electron microscopy which reveals, at high resolution, the cross-corrugated surface texture of a vacuum dried vesicle (Fig. 2C).

Quantitative analysis of the effective Laplace tension, τ, induced by aspiration (eg. Fig. 2A,B) demonstrates the tremendous increase in elastic stiffness and mechanical stability achieved by full, covalent crosslinking of an **OB2** vesicle (Fig. 3A). Prior to crosslinking, **OB2** vesicles respond in a manner generic to fluid phase membranes: they resist aspiration in close proportion to the same interfacial tension, γ, that drives their self-assembly[22]. Thus, like a soap bubble in air, pressurization spheres the outer contour of the fluid vesicle (Fig. 3B lower inset) and homogeneously dilates the membrane's area. The slope of τ versus relative area yields K_a ($=4\gamma$) of 107 ± 14 mN/m (9 vesicles).

Upon crosslinking in a flaccid state (Fig. 3A), **OB2** membranes clearly respond with a more solid-like character in deformation. When aspirated sufficiently, the vesicle contour immediately outside of the micropipette appears flattened (Fig. 2A, B) relative to a sphere (Fig. 3B, lower inset). This flattening results, it seems, from nano-corrugations of buckled membrane that radiate away from the micropipette entrance and stiffen this region against bending (like corrugations in cardboard); such folds are sometimes visible in phase contrast imaging but become most clear when imaged by fluorescence using labeled **OB2** and pressurized vesicles (Fig. 3B upper inset). In general, aspiration curves display three quasi-linear regimes as illustrated and labeled in Fig. 3B. Regime #1, at the lowest pressures, typically involves large deflections of the membrane as dents are smoothed out in forming an initial seal against the micropipette. Regime #2 involves extension and shear of the membrane into the micropipette and is accompanied by circumferential, 'hoop' contraction which likely initiates the radial corrugations. The highest pressure regime #3 invariably appears to smooth out some of the corrugations and likely involves direct dilational work on the covalently cross-linked network, despite no measurable change in surface density (see Fig. 3B caption). The slope of each of these three regimes provides, at

the very least, a phenomenological measure of membrane bending, shear, and dilational resistance; Fig. 4A summarizes these measures as apparent moduli for fully crosslinked **OB2** membranes as well as the crosslink-diluted systems elaborated below.

The remarkable stability of a crosslinked versus a non-crosslinked membrane (Fig. 3B) motivates a chemically controllable approach to property modulation. Notably, **OB2**'s fully hydrogenated homolog of polyethyleneoxide-polyethylethylene (EO_{40}-EE_{37} designated **OE7**)[23] makes membranes which exhibit a nearly identical K_a[16]. This has an important implication: since γ^2 is proportional to the dominant Flory interaction parameter χ that drives self-assembly[24], **OB2** and **OE7** ought to be largely miscible. The miscibility of **OE7** with **OB2** has been confirmed by imaging vesicles containing fluorescently tagged polymers and provides a route to the membrane properties modulation. Dilution of crosslinked **OB2** by blending in **OE7** clearly reduces the apparent stiffness of the composite membrane and, as expected, in linear relation to the crosslinkable mole fraction $X(=X_{OB2})$ (Fig. 4A).

Mixed and crosslinked membranes with X below some critical concentration X_C (~ 16%) of **OB2** are less stable under stress (Fig. 4B). Indeed, the rupture tension τ_R is seen to cross below the baseline value set by pure non-crosslinkable membranes, reaching a minimum measured value near $X \approx 10\%$ that is almost three–fold below baseline (i.e. τ_R of **OE7**). This destabilization is generally reminiscent of that seen with polymerizable lipids[10,11], but structural clues of destabilization mechanism are not so apparent. Confocal microscopy of fluorescently labeled mixtures suggests that, even within the few minutes of the reaction, crosslinking is homogeneous on an optical scale of hundreds of nanometers. This indicates that nano-domain boundaries, as is often the case with phase-separated membranes[25], must be the weak and permeable loci in the membrane. Contrary to the destabilization of the membrane with constituent-limited polymerization, full crosslinking of 100% **OB2** leads to a rupture stress across the membrane that is enormous: $\tau_R/d \approx 1000$ Atm as estimated from osmotic swelling and rupture experiments. This proves

mechanically consistent with crosslink stabilization of the membrane, since phospholipid membranes[25] as well as non-crosslinked polymer membranes rupture in the range of $\tau_R/d \sim$ 10 – 50 Atm. The results above can be used for further theoretical analysis of the crosslinking reaction and associated properties below.

Discussion

Unlike any lipid bilayer, however, polymersome membranes can withstand dilational strains of about 20% before rupture – in comparison to a critical strain of 5% or less that is *universal* to lipid membranes[26]. As a result, transverse strains of about 10%[27,2] that result from free-radical crosslinking can be withstood, allowing nano-structures of copolymer to consistently (and uniquely) survive crosslinking. Consistent with this, phase contrast images clearly demonstrate membrane integrity through sustained retention of small molecule encapsulants. Such a separation of solutes implies a close-packed arrangement of amphiphiles in the membrane: importantly, two dimensional materials, fluid or not, are widely known to fill space with a quasi-hexagonal lattice. In the absence of better structural information, we will therefore assume for modeling purposes below that the copolymer centers-of-mass are arranged on a hexagonal lattice.

Bending and stretching modulus. The membrane bending modulus can be crudely estimated from the direct imaging by scanning electron microscopy which reveals, at high resolution, the cross-corrugated surface texture of a dried vesicle (Fig. 2C). The spontaneous curvature, c, of a typical wrinkle that emerges in the dried state ($c \sim 0.01 - 0.1$ nm^{-1}) may be considered a free energy balance between curvature energy and interfacial tension (Fig. 2C inset). The simplest order-of-magnitude model introduces the bending modulus K_b by combining the curvature energy ($^1/_2\ K_b\ c^2$) integrated over the deformed length $2\pi c^{-1}$, with the work done by the only tension left in the system, the interfacial tension 2γ, that acts over the displacement $(2\pi - 4)c^{-1}$. Neglect of end effects and so-called stretching of ridges [21] will lead to an over-estimate of K_b. We assume that γ for the

vacuum-dried state is approximated by the net difference between the oil-water interfacial tension (implicit in K_a later in the text) and a typical air-water interfacial tension corresponding to the surface energy of EO in vacuum. What results is a crude morphological estimate and upper bound of $K_b \sim 10^{3\text{-}5}$ pN nm. From plate theory[28] then a first approximation for the in-plane streching modulus is $K_b / d^2 \approx 10$ - 1000 mN/m.

Shear modulus. The in-plane shear modulus can be extracted from the micropipette aspiration results. For a pipette radius (R_p) that is smaller than half the effective radius of the aspirated vesicle (R_v), a simple overall measure for extensional strain is the relative aspirated length, $x \equiv L/R_p$, that results from the imposed effective tension, $\tau = \Delta P / 2R_p$. The in-plane shear modulus is then very well approximated by $\mu_{2D} \approx d\tau / dx$. Continuum computations for aspiration of an initially flat, incompressible membrane as described in [29] are readily generalized to an initially curved membrane. These show that μ_{2D} is proportional to $d\tau/dx$ within a factor of about 1.2 – 1.4. For regime #2 (Fig. 3) detailed analyses yield $\mu_{2D} \approx 80 - 120$ mN/m as is separately confirmed by nano-indentation tests with an Atomic Force Microscope (Photos and Discher, unpublished).

Regime #3 provides a measure of the effective dilational resistance. At full crosslinking this is several-fold higher or more than the K_a for the uncrosslinked **OB2** membrane. In addition, the post-crosslinking effective K_a is also several-fold higher than the apparent shear rigidity of regime #2, consistent with a suitable Poisson ratio for a solid-like membrane.

Comparisons with the red cell membrane prove insightful. The spectrin-crosslinked network of the red cell is coupled to a bending-resistant bilayer[30] and exhibits a buckling response in aspiration, like that seen at high pressures with polymersomes here. Preceding this regime of red cell aspiration, however, is an intermediate regime where the spectrin cytoskeleton is strongly stretched in the direction of the pipette axis[29,31]. Nevertheless, compared numerically to a spectrin network with a shear modulus of ~0.01 mN/m, the fully crosslinked **OB2** membrane is obviously far stiffer. The difference undoubtedly reflects the

comparatively higher density of crosslinks, ϕ_X. Nonetheless, a three-dimensional modulus approximated as $\mu_{3D} \approx \mu_{2D}/d$, is at the upper end of reported measures for the elastic modulus of extensively crosslinked polybutadiene. The elasticity thus appears consistent with a highly efficient crosslinking reaction that is not limited by diffusion or damage by free radicals.

Crosslinking efficiency. A projected density of crosslinks, ϕ_X, relates to the shear elasticity via $\phi_X = k_B T / \mu_{2D}$ according to rubber elasticity theory[32,33]. The number of *lateral* crosslinks per copolymer is then $n_{LAT} = {}^1/_2\ \phi_X\ A_C$. The factor of $^1/_2$ reflects a symmetric bilayer of diblocks[16] and A_C represents a projected area per copolymer chain. The minimal area can be estimated from the thickness d, the mass density of bulk PBD, and account for a bilayer structure [16], which altogether yield: $2M_N / \rho_{PBD}\ d \approx 1$ nm^2. Note that meandering of a chain outside of its 'box' gives an overlap with neighboring chains: the maximal area for **OE7** can be as high as 2.5 nm^2 [34] which yields an overlap of linear extent (2.5 nm^2/1 nm^2)$^{1/2}$ = 1.6. This implies that contact interactions with *second* neighbor chains will be frequent. Given the projected area range, the number of *lateral* crosslinks per copolymer is calculated to be 12 – 31. Provided the same density of crosslinks acts to interconnect the two lamellae, the number of interlamellar or *vertical* crosslinks per copolymer is estimated as $n_{VERT} \approx d^{-1}\ (A_C/\pi)^{1/2}\ n_{LAT} \approx 1 - 3$. A finite interlamellar crosslinking such as this is more than enough to prevent chloroform inclusions from forming within the copolymer bilayer (Fig. 2A). Moreover, an overall efficiency for intermolecular crosslinking is broadly estimated by normalizing against the number of butadiene units as $(n_{LAT} + n_{VERT}) / n \approx 30 - 75\%$, with the higher efficiency being most consistent with measures of A_C. The remainder of reacted bonds must therefore be internal to each copolymer. The less-than-perfect efficiency underscores the importance of designing in 'redundant' chain reactivity.

Rigidity percolation through a nano-stack. The tightly interconnected nature of the crosslinked **OB2** membrane as well as its obvious integrity as a permeability barrier

(Fig. 1C) reinforce the idea of a close-packed arrangement of copolymers. In projection, this translates into a hexagonal arrangement of copolymers skeletonized as a triangular lattice (Fig. 3C upper inset). Within this (initially) liquid crystalline lattice, a simple topological model emerges for the crosslinking that governs the elastic stretching response: the depth d is proposed to be divided into N_{elas} layers such that, within each layer, each copolymer participates in six crosslinks with its neighbors. For a triangulated network of polymer tethers, μ is proportional to the average effective elastic constant of constituent tethers or springs [35]. Projecting onto a plane a finite stack of N_{elas} such network tethers or springs, in register, is equivalent to summing up the springs in parallel. The effective elastic spring constant, k, is then simply related to the effective elastic constant in each layer through: $k = N_{elas}\, k_{LAYER}$. Random elimination of tethers – with the projected network still intact – will result in a continuous, linear reduction of k, and thus μ. Similar linear scaling should apply to rupture processes since tether forces are also proportional to k_{LAYER}. The number of layers per lamellae is then given by $^1/_2\, N_{elas} = n_{LAT} / 6 \approx 2 - 5$.

Although the linearity of the rupture energy with respect to the mol fraction of the polymerizable component is consistent with rubber elasticity theory, the extrapolated offset by a critical fraction X_C =16±2% appears non-trivial. For a triangulated network of central force interactions a rigidity percolation limit of $p_C = {}^2/_3$ is readily derived by mean-field arguments[36,37]: most simply, one of three legs removed from a triangle is sufficiently destabilizing. Combining such destabilization with the extensive crosslinking along each polybutadiene segment suggests a stack of bond-depleted networks. Each BD block is viewed as perforating N_C layers, thereby forming a node in a stack of triangulated networks (Fig. 3C inset). Progressively severing all but 6 of the $6N_C$ elastic tethers emanating from each copolymer, the stack is expected to exhibit a linear decrease in its elastic constants as it is reduced in projection to a single triangulated network. Further dilution also leads to a linear decrease in the elastic constants – within mean-field theory – down to the critical fraction

$$X_C = p_C\,(6 / 6\,N_C). \qquad (1)$$

For X above X_C the membrane is solid-like, and for X well below X_C the membrane is fluid-like; these expectations are confirmed by fluorescence photobleaching experiments to be reported elsewhere (Lee et al, in preparation). Note that the dependence of X_C on lattice-connectivity is manifested *only* in the prefactor, p_C, and thus reduces the sensitivity to the presumed close-packed, quasi-hexagonal symmetry. Solving Eq. 1 yields $N_C = 4 - 5$ which is in very good agreement with N_{elas} despite the very different mean-field physics. The vision of stacked percolating networks is fortified below by measures of the dependence of the membrane rupture tension, τ_R, on X above X_C.

Membrane cracking for $X >> X_C$. Between X_C and the fully crosslinked limit, the rupture stress is found to scale approximately as $(X - X_C)^\beta$, $\beta \approx 1.48$. This will be shown largely consistent with a simplified, planar form of Griffith crack theory – where interface energy competes against bulk energy. In terms of suitable D-dimensional parameters and fields[Arndt, to appear 2001 #11], the cost of forming a rupture interface of energy density γ_R is balanced against the elastic stress energy, $\sim\sigma_R^2/\mu_D$, that is relieved upon rupture. Note that, for D=2, γ_R has units of energy per length and $\sigma_R \equiv \tau_R$. For a so-called penny crack of width w in D-dimensions, the rupture energy is $\varepsilon_R = \gamma_R\, w^{D-1}$, and the total free energy, ignoring constants, has the form $F \approx \gamma_R\, w^{D-1} - \sigma_R^2 / \mu_D\, w^D$. Minimization with respect to w yields:

$$\sigma_R = constant \times (\varepsilon_R \mu_D / w^D)^{1/2} \qquad (2)$$

While $\varepsilon_R \sim (X - X_C)^{D-1}$ is reasonably intuitive, rubber elasticity theory suggests and Fig. 3C confirms (for D=2 at least) that $\mu_D \sim (X - X_C)^1$. Furthermore, a simple scaling for the crack width is given by the average inter-nodal spacing of the crosslinked structure as $w \sim (X - X_C)^{-1/D}$. Combining these separate scaling relations yields $\sigma_R \sim (X - X_C)^\beta$, where $\beta = (D+1) / 2$. Clearly, $D = 2$ fits the experiments and confirms that the crosslink-dependent rupture tension reflects a small number of layered-crosslinks compared to the large number being stressed.

Chains of crosslinked OB2 in a homogeneous fluid of OE7 for $X < X_c$. The rupture tension was found to be a minimum at a measured value of 10 mN/m near $X \equiv X^* =$ 10%. Interestingly, the decrease in τ_r from $X = 0$ to X^* is linear ($R^2 = 0.997$) with a slope of (1 mN/m) per $\%X_{OB2}$. Within this same range, Fig. 4A suggests that the membrane is overall fluid, devoid of any shear elasticity. Furthermore, the noted lack of any optically-resolvable domain structure (with fluorescently tagged copolymer) suggests a homogeneous phase. Based on these results for $0 \leq X \leq X^*$, we propose a solid-in-fluid nanophase that has a lateral interface between the two phases which facilitates or nucleates rupture. This incompatability which we suggest emerges could well reflect crosslink-strain[2] induced mismatch of hydrophobic core thicknesses. Since this interface evidently grows in linear proportion to crosslinked mass, linear or ramified chains of crosslinked copolymer are implicated as opposed to tight, separated domains. This picture is certainly consistent with the initially high miscibility between **OB2** and **OE7**. Moreover, as these chains begin to percolate across the entire surface as X increases from 0 to X_c, the elastic rigidity and toughness ought to suddenly increase, just as found (Fig. 4). Most dramatic perhaps is the rapid rise in τ_r that is sketched between ~10 – 15% **OB2** (i.e. X^* and X_c) and is highly suggestive of critical phenomena. While focused on copolymer, the mechanism suggested here highlights the limitations of polymerizable lipids with minimal reactivity as well as the importance of intrinsic membrane robustness.

Summary

Covalent crosslinking of nano-structures that self-assemble in solution is rapidly emerging as a way of making permanent what is, by definition, labile. Establishing the efficiency as well as the physical effects of cross-linking is not trivial at such scales, however. It is nonetheless important because intermolecular polymerization competes – unsuccessfully if reactivity is limited – against intramolecular bond formation as well as other mechanisms of termination. Stability tests reported here for a polymer membrane

provide very direct evidence of thorough and durable cross-linking over length-scales of microns. Since defects as small as a sucrose molecule would be rapidly revealed through permeation and stress-sensitive rupture, the proof here appears robust. What is thus most clear is that efficient cross-linking is possible and tunable through blending at the nanoscale, with dramatic effects on strength and durability and a definitive transition from interface-dominated properties to crosslink-dominated. In addition, such structure-constrained crosslinking inspires new views of phenomena such as rigidity percolation in reduced dimensionality systems.

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Figures

Fig. 1. Formation and osmotic deflation of **OB2**, i.e. $(EO)_{26}$-$(BD)_{46}$, vesicles either without (A, B) or with (C) crosslinking between the hydrophobic segments of butadiene. (A) Brightfield image of bulk copolymer hydrating and vesiculating into a 200 mOsm sucrose solution. Giant, spherical, unilamellar vesicles predominate. The scale bars for A, B, and C are all 5 μm. Separate cryo-TEM images show the hydrophobic core thickness to be $d = 9\pm1$ nm[26]. (B) Dilution into 300 mOsm phosphate-buffered saline (PBS) increases the internal osmotic pressure by deflating a spherical, non-crosslinked vesicle. A smooth vesicle contour is generated, and the refractive index difference between sucrose and PBS makes vesicles appear dark in phase contrast imaging, proving the integrity of the vesicle's membrane. (C) When crosslinked and subsequently diluted into PBS, the vesicle deflates with dents and wrinkles, characteristic of a solid-like membrane. (D) Illustrative schematic of the diblock copolymer membranes, showing a close-packed arrangement of copolymers with a crosslinked core thickness d.

Fig. 2. Stability of individual vesicles of crosslinked **OB2** during transfer into chloroform (A), air (B), or vacuum (C). (A) The top two images show a sucrose-containing vesicle held by a micropipette (of inner radius R_p = 4.8 μm) in PBS solution and viewed in phase contrast (dark) or brightfield. The middle image shows a vesicle transferred into chloroform and held there for 30 min, followed by transfer back into aqueous solution. Sucrose retention is visually obvious from the phase contrast in the first and last images. This is confirmed by the last image which shows the expected decrease in vesicle volume when the exterior osmolarity is increased. (B) A vesicle in aqueous solution pulled into a micropipette (R_p = 4.2 μm) and then removed from the chamber and imaged within seconds after exposure to air. Rehydration occurs immediately upon reinsertion of the vesicle back

to the aqueous solution. (C) Scanning electron micrograph of a vesicle prepared simply by vacuum drying without staining or fixation. The long axis of the vesicle is 10 μm. The sketch represents tension-driven wrinkling.

Fig. 3. Deformability of crosslinked **OB2** vesicles in comparison with non-crosslinked **OB2**. (A) An **OB2** vesicle that was crosslinked in a highly deflated (non-spherical) state. Manipulative rotation shows the dimpled, red cell-like shape. (B) Further aspiration into a micropipette generates a membrane tension that is proportional to the applied pressure. Extension to a length L into the pipette depends, however, on the compliance of the membrane as well as the vesicle's volume, V, relative to a sphere, V_{MAX}, of the same area. When vesicles are aspirated without crosslinking, the tensions up to rupture are orders of magnitude smaller than the ~1000 mN/m sustainable by a crosslinked membrane. Also note that, without crosslinking, the membrane contour outside of the pipette appears spherical, consistent with a tensed fluid surface. Once crosslinked, aspiration of a flaccid vesicle ($V< V_{MAX}$) is more complex, with three regimes of response. At small extension (denoted regime 1), the membrane appears to seal against the micropipette as large 'dents' are smoothed out. At an intermediate extension (regime 2: $L \approx R_p$), the outer vesicle contour appears more axisymmetric with the membrane forced to constrict upon entering the pipette; this is the regime most readily identified with membrane shearing. In a final high pressure regime 3, extension into the micropipette is minimal while wrinkles appear locked in by contact with the pipette wall (B, top image). The slopes, $d\tau/dx|_i$ of each regime provide respective measures of effective moduli. Notably, $d\tau/dx|_2$ is related to μ_{2D} as explained in the text, and $d\tau/dx|_3$ is related to a nominal K_a. For the latter, one can show, within a factor of about two and provided shear is neglected, $K_a \approx 20\, d\tau/dx|_3$, at least within the experimentally relevant range of R_v/R_p ($\approx$ 1.2–3)[29]. For the crosslinked membranes, length increments with pressure changes are extremely small and imply that $K_a >> 10^2$ mN/m.

Fig. 4. Elasticity and mechanical stability of crosslink–diluted **OB2** vesicles made by blending in varying molar ratios of the non-crosslinkable analog **OE7**. (A) The three effective elastic constants illustrated in Fig. 3 scale linearly with crosslink dilution above a critical fraction of **OB2**. Linear fits all intersect at a mole fraction of X_{OB2} = 16±2% near E_{app} = 0. (B) The effective rupture tension, τ_R, is a more non-linear function of crosslink dilution. Above X_{OB2}≈15% (≡X_C), τ_R increases monotonically with weak power-law scaling (lower inset). Below X_C, τ_R exhibits a minimum: the entire gray region highlights crosslink-induced destabilization. Importantly, the crosslinking reaction does not alter the previously reported value of τ_R^o for a pure **OE7** system. In addition, we followed the kinetics of all crosslinking reactions through measurements of τ_R and find that polymerizations are complete within 10 minutes of initiation; values of τ_R reported here correspond to end-point measures. Each data point represents an average (± S.D.) of measurements on at least 3 vesicles.

Fig. 1

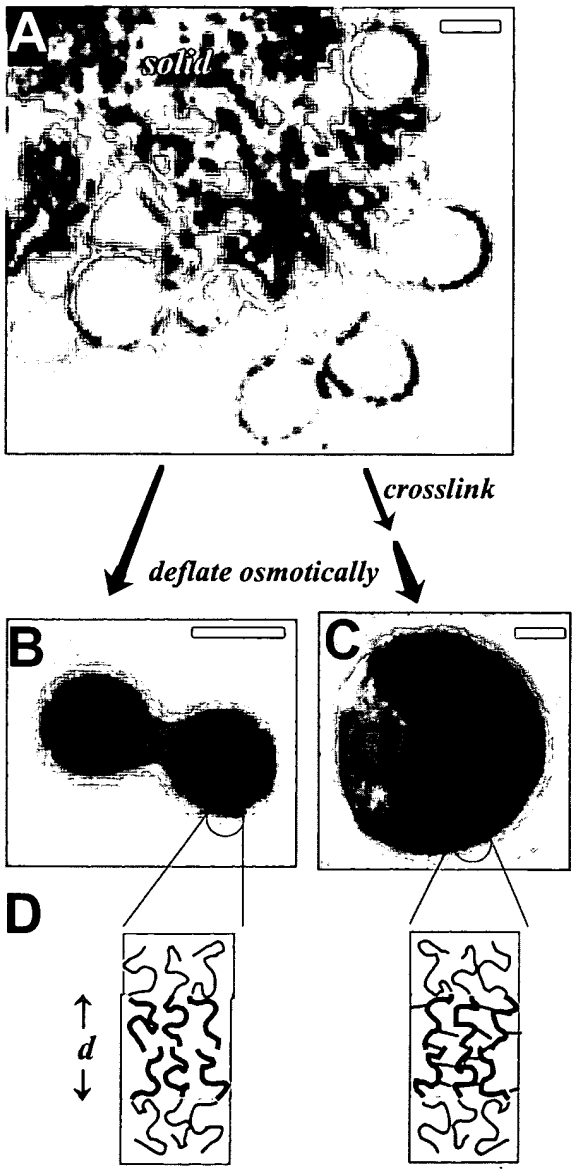


Fig. 2

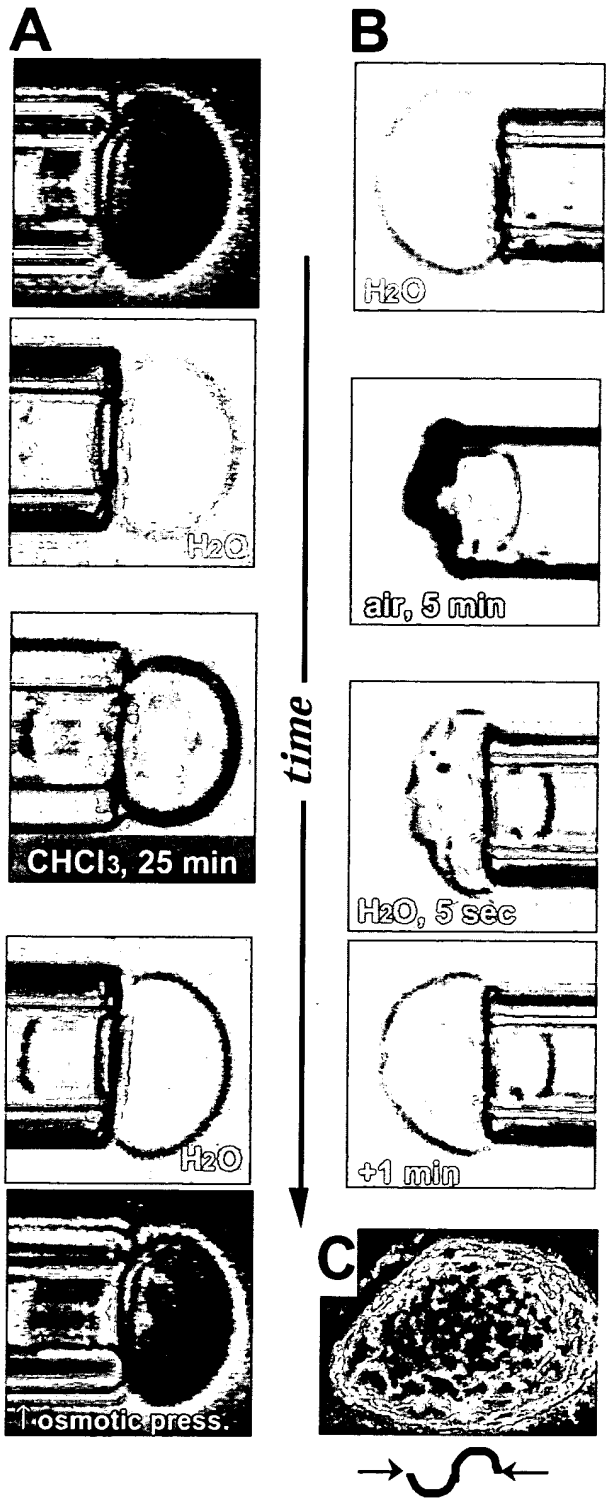

A
B
H2O
H2O
air, 5 min
CHCl3, 25 min
time
H2O, 5 sec
H2O
+1 min
↑osmotic press.
C

Fig. 3

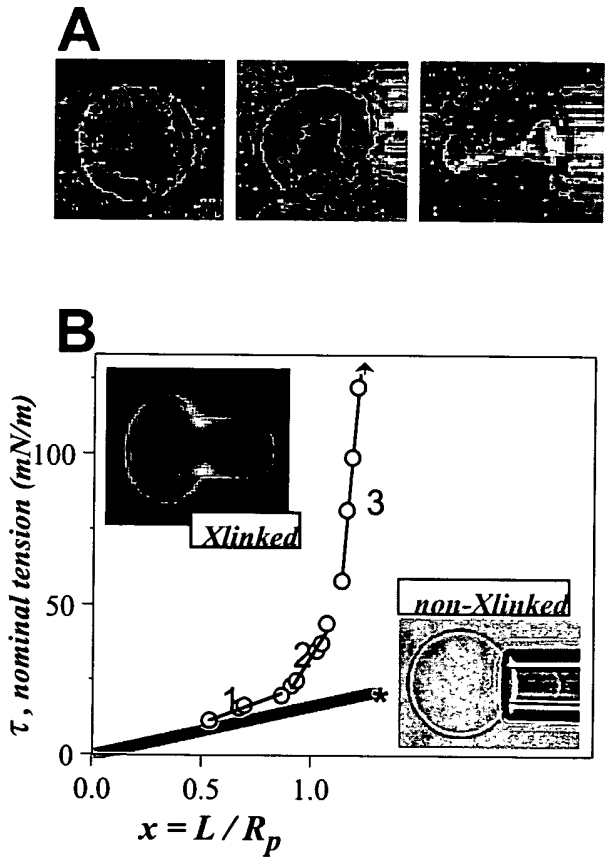
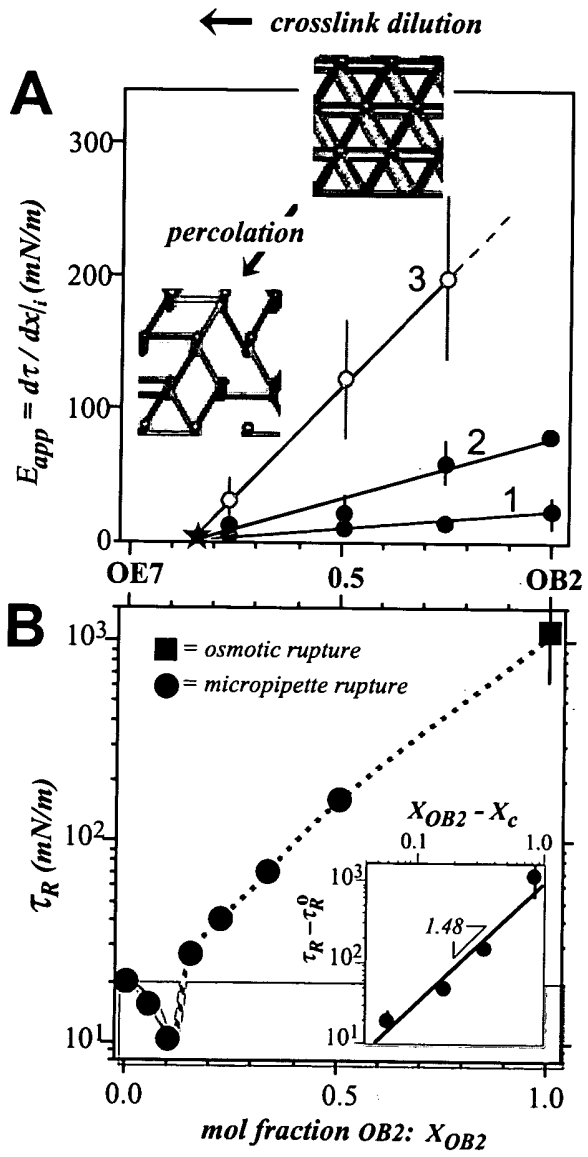

A
B
Xlinked
non-Xlinked
1
2
3
100
50
0
0.0
0.5
1.0
τ, nominal tension (mN/m)
x = L / R_p

Fig. 4.


crosslink dilution
A
300
200
100
0
$E_{app} = d\tau / dx|_i$ (mN/m)
percolation
3
2
1
OE7
0.5
OB2
B
10^3
10^2
10
τ_R (mN/m)
■ = osmotic rupture
● = micropipette rupture
$X_{OB2} - X_c$
0.1
1.0
$\tau_R - \tau_R^0$
1.48
0.0
0.5
1.0
mol fraction OB2: X_{OB2}